APPENDIX C

CASE STUDIES
(A, B, C, D, E)

Framework for Human Health Risk Assessment Colloquia Series

Colloquium #1

CASE STUDY "A"

1.0 INTRODUCTION

Compound A is a common low molecular weight halogenated compound. It is found in water as a common byproduct of chlorination and occasionally due to contamination from its use as a solvent. Its presence in water can lead to significant oral and dermal exposures. Inhalation is also a significant exposure concern as a result of volatilization from water or pure solvent.

As pure compound, Compound A is a volatile liquid that is denser than water and sparingly soluble. It is relatively slowly reactive (i.e., relatively stable), requiring enzymatic catalysis in the body or exposure to heat, light, and oxygen for reactivity in the environment or in industrial use. Compound A vapor is classified by EPA as a Category 3 gas (low water solubility and low reactivity).

Compound A causes central nervous system, renal, and liver noncancer toxicities in humans and laboratory animals following acute and chronic exposures. It causes nasal toxicity in rodents. In animals, it causes tumors of the liver and kidney. This case study focuses on the toxic and carcinogenic actions on the nasal passage, kidney, and liver from chronic inhalation and oral exposure of rodents to Compound A.

2.0 TOXICOKINETICS

Compound A, like many low molecular weight chlorinated compounds, is readily absorbed by inhalation and oral exposures. Significant kinetic differences in absorption from aqueous versus oil solutions have been reported. It is subject to saturable metabolism primarily by cytochrome P450 2E1. Due to the saturable metabolism, the parent compound is exhaled at high doses regardless of the route of exposure. The major metabolite eliminated by the body is carbon dioxide.

Cytochrome P450 2E1 is present in the liver, kidney cortex, and respiratory tract tissues (tracheal, bronchial, olfactory, and respiratory nasal mucosa; and esophageal, laryngeal, tongue, gingival, cheek, nasopharyngeal, pharyngeal, and soft palate mucosa) of rats. Autoradiography studies in rats demonstrate a good correlation between tissue adducts of Compound A and metabolic capability. Though more limited information is available for mice and humans, similar distributions of 2E1 are observed. Quantitative studies, however, show that human nasal tissue has approximately 10% of the metabolic capacity of rodents.

Metabolism by the oxidative pathway forms an alcohol that spontaneously dehalogenates to form a highly unstable ketohalogen. This compound reacts with water to form carbon dioxide and acid (HX). Alternatively, Compound A reacts with any available cellular nucleophile, resulting predominantly in glutathione, lipid, and protein adducts. Glutathione depletion can occur at high doses, leading to greater cellular damage. Due to factors such as glutathione depletion and saturation of metabolic pathways, quantitative differences among species or different dose routes, vehicles, and exposure regimens need to be evaluated to provide a consistent understanding of observed toxicities. By accounting for kinetic differences, the role of alterations in the toxicity process (i.e., pharmacodynamics) from factors such as corn oil versus aqueous solution can be evaluated.

Reductive metabolism of Compound A has been demonstrated *in vitro* using anaerobic incubations. Under normal oxygen tension, free radical formation by isolated hepatocytes was reduced but not eliminated. It has been shown that maximal lipid peroxidation from reductive metabolism of Compound A occurs at 10 mm Hg oxygen tension because of opposing requirements for oxygen; low oxygen increases reductive metabolism, but oxygen is required to propagate the lipid peroxidation reaction sequence.

3.0 EFFECTS IN HUMANS

Reports from intentional human exposures demonstrate acute responses similar to those observed in animals. Central nervous system depression and cardiac arrhythmias occur following inhalation of high concentrations. Liver toxicity has been reported following an oral poisoning episode and inhalation of anesthetic concentrations. Renal tubular necrosis and renal disfunction have also been reported following inhalation of anesthetic concentrations.

Epidemiological studies of occupationally exposed workers have reported limited evidence of liver toxicity generally described as toxic hepatitis. Studies of chlorinated drinking water consumption and cancer have provided limited associations with urinary bladder and colon cancer and low birth weight. However, because of the presence in chlorinated drinking water of a substantial number of chlorination by-products, the association between Compound A itself and reproductive and/or cancer toxicity is unclear.

4.0 EFFECTS IN ANIMALS

4.1 Nasal Toxicity

Nasal passage toxicity has been observed in rodents following both oral and inhalation exposure, suggesting a systemic response to bloodborne Compound A. Nasal toxicity increases in a dose dependent manner; it occurs at lower doses or concentrations than any other target organ toxicity. In contrast to the other two target organs, no tumors were observed in the nose in any of the chronic assays with rats or mice.

Inhalation exposures of F344 rats produced nasal toxicity, the type and severity of which were dependent upon the exposure concentration (0, 2, 10, 30, 90, 300 ppm) and duration (4 days, 3,

6, and 13 weeks). The lesions, like those observed following oral exposure, were in specific regions of the nasal ethmoid turbinates of both males and females. Following 4 days of exposure, observations included edema, loss of deep Bowman's glands, periosteal hypercellularity, and new bone growth in portions of the ethmoid turbinates. Focal atrophy of the olfactory epithelium was noted in rats exposed to 90 and 300 ppm. The most prevalent lesion in rats exposed to at least 10 ppm for 3 weeks was loss of deep Bowman's glands and edema in the lamina propria. Following exposures of 6 and 13 weeks, atrophy of the ethmoid turbinates was noted, minimally at 2 ppm and increasing in severity with dose. Labeling index studies found large increases at 10 ppm and higher concentrations following 4 days of exposure. By 3 weeks, labeling had dropped significantly and continued to drop to 13 weeks, although control levels were never attained.

Following oral exposures of female F344 rats, two treatment-related responses were observed in specific regions of the nasal passages, referred to as peripheral and central. Peripheral toxicity included new bone formation, periosteal hypercellularity, and increased cell replication. Following a 3-week exposure, the severity was dose dependent, with minimal changes at 34 mg/kg/day increasing to moderate severity at 400 mg/kg/day (all effects were statistically significant). Central toxicity following 4 days of exposure included degeneration of the olfactory epithelium and superficial Bowman's glands at the highest dose (400 mg/kg/day) and only individual cell loss at the lower doses (34, 100, 200 mg/kg/day). Following 3 weeks of exposure, there was substantial regeneration of the olfactory epithelium; no lesions remained at 34 mg/kg/day. Cell proliferation in the nasal turbinates increased with dose following both 4-day and 3-week exposures at 24 and 100 mg/kg/day, respectively, but little further increase in proliferation occurred at higher doses.

Although some lesions observed in mice were similar to those in rats, they were not identical. Early proliferative lesions were transient, and a late atrophic response was not apparent in the mouse.

4.2 Kidney Toxicity

Increased kidney tumors have been observed in mice and rats exposed chronically.

Male ICI mice exposed orally to 0, 17, and 60 mg/kg of Compound A for 104 weeks had increased adenomas and carcinomas (0/72, 0/37, and 8/38) only at the highest dose, and no increase in females was observed. Inhalation exposure also resulted in tumors in male but not female BDF1 mice. In males, combined adenomas and carcinomas increased at the top two concentrations (0/50, 1/50, 7/50, and 12/48 for 0, 5, 30, and 90 ppm exposures, respectively).

In two studies with OM rats exposed by corn oil gavage and drinking water, an increase in kidney tumors in males was observed. One study also exposed females and a single tumor was seen in the high-dose group. In an oral study with male and female Sprague-Dawley rats (0, 15, 75, and 165 mg/kg) and the inhalation study with F344 rats (0, 10, 30, 90 ppm) no tumors were observed.

Renal tubule injury, cell proliferation, and other cellular and tissue responses to injury were observed in both mice and rats following exposure to Compound A. These effects were observed at the doses used in the cancer bioassays and are observable in tissues that also have neoplasms. Histopathological evaluation of kidneys from a positive rat bioassay, for instance, found evidence

of proximal tubule cytotoxicity. Cell injury involved vacuolation, necrosis, and nuclear enlargement affecting the proximal convoluted tubule of the cortex. Injury was observed in males, but not females, of several mouse strains. Less complete information is available for rats, and much of it is in strains for which there are no cancer data. Kidney damage has been observed in rats, and sex differences appear less pronounced than in mice.

4.3 Liver Toxicity

Compound A has been evaluated for noncancer and cancer effects in rats and mice exposed by the oral and inhalation routes. Under specific exposure conditions, it causes liver and kidney tumors as described in this and the previous sections. Noncancer effects were observed in the liver and kidney in both species, as well as the previously described nasal toxicity.

Noncancer Effects: Hepatotoxicity in various animal species exposed by inhalation has been reported in several studies. Serum sorbitol dehydrogenase (SDH) activity was increased in rats exposed to 153 ppm and above for 4 hours in one study, and SGPT levels were increased in mice exposed to 100 ppm, 7 hour/day for 8 days during various stages of pregnancy in another study. These increased enzyme levels in serum indicate hepatocellular necrosis. Fatty changes were observed microscopically in male and female mice after acute exposure to Compound A concentrations of 100 ppm. Liver necrosis was observed in female rats exposed to 4,885 ppm Compound A for 4 hours and in male mice that died after acute exposure to 692 to 1,106 ppm Compound A, but not in those that survived and were terminated after a 12-month recovery period, indicating that the liver damage was reversible. Centrilobular granular degeneration was observed in rats, rabbits, and guinea pigs exposed to 25 ppm Compound A for 6 months, but not in dogs exposed to 25 ppm for the same time period; however, these pathological findings were not observed in the 50 ppm exposure group of rabbits and guinea pigs or the 85 ppm exposure group of guinea pigs. Although the liver effects in rabbits and guinea pigs were not dose-related, the small number of surviving animals in the higher exposure group may have biased the results of the study and may not fully describe the pathological effects of Compound A at the higher dose.

The liver is also a target organ for Compound A oral toxicity in animals. In acute studies, increased serum levels of transaminases, indicative of liver necrosis, were observed in mice treated with a single gavage dose of 273 mg/kg in oil or 250 mg/kg/day in oil for 14 days. Centrilobular necrosis of the liver with massive fatty changes was also observed in mice after a single dose of 350 mg/kg Compound A in oil. At a dose of 35 mg/kg, minimal lesions consisting of midzonal fatty changes were observed in mice.

Liver effects in animals have been reported in numerous oral studies of intermediate duration. Female mice were exposed to 3, 10, 34, 90, 238, and 477 mg/kg/day of Compound A in corn oil via gavage for 5 days per week for 3 weeks. Compound A treatment resulted in significant increases in liver weights of mice at 90, 238, and 477 mg/kg/day and 34 mg/kg/day resulted in pale cytoplasmic eosinophilia of the centrilobular hepatocytes and mild vacuolation of the centrilobular and midzonal hepatocytes relative to the periportal hepatocytes and livers from control mice. At the 238 mg/kg/day dose, the livers were characterized by a severe centrilobular hepatocyte necrosis. At 477 mg/kg/day, the central zone of the liver was populated by degenerate vacuolated hepatocytes and regenerating hepatocytes with markedly basophilic cytoplasm and small round nuclei with clumped chromatin and prominent nucleoli. Significant dose- dependent

increases in ALT and SDH were observed at doses of 34 mg/kg/day and greater. Cell proliferation was markedly increased in the liver at the 238 and 477 mg/kg/day doses. Mice dosed with 16, 43, 82, 184, or 329 mg/kg/day of Compound A in the drinking water for 7 days a week for 3 weeks showed in no histological changes in livers at all doses studied. Liver weights were significantly increased at 82, 184, and 329 mg/kg/day.

Another study examined the dose response relationships for the induction of cytolethality and regenerative cell proliferation in the livers of male Fischer 344 rats given Compound A by gavage. Groups of 12 rats were administered oral doses of 0, 3, 10, 34, 90, and 180 mg/kg/day Compound A in corn oil by gavage for 5 days per week for 3 weeks. BrdU was administered via an implanted osmotic pump to label cells in S-phase. Cells having incorporated BrdU were visualized in tissue sections immunohistochemically and the LI evaluated as the percentage of S-phase cells. Necropsies and histopathological examinations were performed at death. The relative liver weights were increased at doses of 90 mg/kg/day and greater at 3 weeks. After 3 weeks of exposure, livers of rats in the 34 or 90 mg/kg/day dose groups did not differ from controls. In the 180 mg/kg/day dose group, effects were similar to those seen at 4 days after exposure. Dose-dependent increases in both ALT and SDH were observed after 3 weeks in the 180 mg/kg/day dose group only.

The toxicological effects of Compound A administered in the drinking water in rats were studied. Groups of 12 rats were administered Compound A in drinking water at concentrations of 0, 60, 200, 400, 900, and 1,800 ppm for 7 days/week for 3 weeks. BrdU was administered via an implanted osmotic pump to label cells in S-phase. Cells having incorporated BrdU were visualized in tissue sections immunohistochemically and the LI evaluated as the percentage of S-phase cells. Necropsies and histopathological examinations were performed at death. Average daily doses of Compound A ingested from drinking water were: 0, 6.0, 17.4, 32.0, 62.3, and 106 mg/kg/day for 3 weeks of exposure for 0, 60, 200, 400, 900, and 1,800 ppm concentration levels, respectively. Only mild hepatocyte vacuolation was observed in rats given 900 or 1,800 ppm in water for 3 weeks. No increase in the hepatic LI was observed at any time.

Fatty changes, necrosis, increased liver weight, and hyperplasia have been observed in rats exposed to 150 mg/kg/day Compound A via gavage for 90 days. Fatty and hydropic changes, necrosis, and cirrhosis were observed in mice treated by gavage with 50 mg/kg/day Compound A in oil for 90 days or 86 mg/kg/day in drinking water for 1 year. In contrast, centrilobular fatty changes observed in mice at 64 mg/kg/day Compound A in drinking water for 90 days appeared to be reversible, and no liver effects were found in mice treated with 50 mg/kg/day Compound A in aqueous vehicles.

In chronic exposure studies, liver effects have been observed in rats, mice, and dogs after oral exposure to Compound A. Necrosis was observed in female rats treated by gavage with 200 mg/kg/day Compound A in oil for 78 weeks. Nodular hyperplasia occurred in all groups of male and female mice similarly treated at 138 mg/kg/day. Fibrosis of the liver was observed in both sexes of rats exposed to 200 mg/kg/day Compound A in the drinking water for less than 180 weeks. Increased SGPT was observed in dogs given Compound A in toothpaste capsules for 7.5 years. The lowest oral dose administered to animals in chronic studies was 15 mg/kg/day, which increased SGPT in dogs.

***Cancer Effects**:* A chronic study of B6C3F1 mice exposed to Compound A in corn oil gave the largest increases in liver neoplasms using high doses (time-weighted averages of 138 and 277 mg/kg for males and 238 and 477 mg/kg/day for females). Observed incidences of hepatocellular carcinomas were 1/18, 18/50, 44/45 and 0/20, 36/45, and 39/41 for control, low- dose, and high-dose males and females, respectively. By contrast, a drinking water study with female B6C3F1 mice exposed to time-weighted average doses of 0, 34, 65, 130, and 263 mg/kg found no increased tumor incidence despite using a dose equal to a positive dose in the corn oil gavage assay. A study using orally exposed (0, 17, 60 mg/kg) ICI mice found no effect in males or females, though a second group of males exposed using a different vehicle showed an increase. An inhalation study using BDF1 mice exposed to 0, 5, 30, or 90 ppm Compound A found no statistically significant increases in adenomas, carcinomas, or combined tumors though a trend analysis was positive for the combined neoplasm rates.

There are five chronic studies using four strains of rats exposed by corn oil gavage, drinking water, and inhalation. These studies were negative or showed marginal increases in hepatocellular neoplasia that were not statistically significant, even when doses were similar to those used in mice. One drinking water study appears positive with 0/18 adenomas in control females and 10/40 in treated female Wistar rats. However, this study is difficult to interpret for several reasons: i) exposed females lived about 185 weeks versus only 145 weeks for controls, and ii) the number of control animals is small, making the incidence more uncertain.

The positive results in mice with corn oil gavage and the negative findings in mice exposed through drinking water raises questions about the appropriate dose metric for dose-response assessment. Neither the daily dose nor the cumulative dose of Compound A are predictive of the tumor outcome. Results from several studies suggest that the greater toxicity with corn oil gavage is due to some combination of pharmacokinetic and pharmacodynamic factors.

5.0 ADDITIONAL DATA RELEVANT TO MODE OF ACTION

5.1 Genotoxicity

More than 40 studies using *in vitro* and *in vivo* assays for a large number of endpoints indicative of various kinds of DNA damage have been undertaken with Compound A. Studies have looked at a variety of endpoints, including those associated with direct or secondary DNA damage. Direct DNA damage endpoints included mutation (i.e., point mutations, small insertions, or deletions), clastogenicity, recombination, sister chromatid exchange, DNA breakage, and DNA adduct formation. Secondary damage endpoints included DNA repair, cell transformation, and anueploidy. The results for mutagenicity assays and sister chromatid exchange are briefly summarized here as representative of the kind of results obtained for most of the endpoints.

Mutagenicity studies for Compound A have been conducted primarily in bacteria (*S. typhimurium*, *E. coli*, *Photobacterium*) with additional studies in yeast, Aspergillus, and cultured chinese hampster V79 cells. Clear positive results were obtained in the studies with *Photobacterium* and yeast. One study used bacteria bioengineered to express glutathione transferase theta which has been implicated in the genotoxicity of related compounds, including methylene chloride and bromochloromethanes. This study was clearly negative with Compound A. In vivo studies included two *Drosophila* sex-linked recessive lethal assays, which were both negative, and two host-mediated assays with bacteria, of which one was positive. Although yeast appear susceptible to Compound A, these results overall appear strongly negative across a range of species.

Sister chromatid exchange is a very sensitive indicator of chemical effects on DNA, although its relationship to carcinogenesis remains unclear. Of the seven *in vitro* studies, four were positive, including one using plant cells. Studies using mammalian cells and chinese hamster ovary (CHO) and human lymphocyte cultures gave equal numbers of positive and negative responses. The one *in vivo* study of male mice exposed to 200 mg/kg Compound A for 4 days reported a statistically significant increase in SCEs in bone marrow cells. Notably, none of these assays use cells from the two organs where tumors are reported. A range of factors can contribute to the mixed results. Compound A is volatile, so *in vitro* assays done in closed containers are preferable to those in open systems. Formation of genotoxic compounds may arise due to the use of stabilizers, even in highly purified preparations of the compound.

5.2 Metabolism and Cell Proliferation in Kidney Tissue

Metabolism is one major factor leading to the variations between sexes. Cytochrome P450 2E1 is present in kidneys of mice and rats, with the highest levels in the proximal convoluted tubules, the site of toxicity. Several studies demonstrate a correlation between levels of covalently bound radiolabel derived from Compound A (an indicator of metabolic activity) and kidney tissue damage. Order of magnitude differences in bound radiolabel have been demonstrated between males and females; two-fold differences were shown between strains with differing susceptibility to neoplasms. The sex differences are under hormonal control, as demonstrated by reduced radiolabel binding and nephrotoxicity in castrated males and increased binding and renal injury in testosterone-treated females. There also appear to be differences in tissue sensitivity; a neoplasm-susceptible strain of mice had greater radiolabel accumulation in kidney compared to a nonsusceptible strain, even after correcting for the higher metabolism in the susceptible strain.

Quantitative studies of cell proliferation in the kidney have been carried out in mice and rats. In the mouse strain used for the inhalation bioassay, for instance, 7- to 10-fold increases in labeling index were observed in males but not females following 4-day exposure to 30 and 90 ppm; no change was observed at 5 ppm. These results correlate with the observation of tumors in the chronically exposed high-dose males. Other studies have shown cell proliferation to vary over dose, exposure duration (decreasing in low-dose groups and continuing in high-dose groups), and exposure route (e.g., no increase with drinking water, but increased with corn oil gavage). These studies are in a variety of species that are untested for cancer or nonsusceptible to kidney tumors, so the results provide a general perspective but are not directly applicable to the cancer studies.

5.3 Cellular Damage and Repair in the Liver

A highly reactive metabolite of Compound A is formed by enzymes of the endoplasmic reticulum and reacts with water, soluble nucleophiles on small molecules (e.g., glutathione) and macromolecules (e.g. proteins), and macromolecular constituents of nearby organelles (e.g., lipids, proteins). Over time, this damage can lead to other damage (e.g., to DNA or organelles dependent upon normal cell function) and becomes histologically observable as necrosis and atrophy. The response to cellular damage includes repair processes in cells, cell proliferation by other cells in the tissue, and tissue repair (e.g. immune cell clearance of damaged tissues). Studies *in vivo* have found cell proliferation to be dependent upon a range of pharmacokinetic and pharmacodynamic factors, including dosing vehicle (corn oil versus aqueous gavage), exposure regimen, strain, and species.

As described for kidney toxicity, there are studies strongly supporting the correlation of metabolism with liver toxicity. CYP2E1 levels are highest in centrilobular regions of rats and humans, the region of greatest damage from ethanol (a 2E1 inducer) and halogenated alkanes. Further, GSH levels are lower in centrilobular regions, likely contributing to observations of GSH depletion following high oral doses.

Histological observations in exposed livers vary with dose, exposure duration, and strain/species. Effects include fatty infiltration, glycogen depletion, cytotoxicity, and necrosis. Induction of cytotoxicity and regenerative cell proliferation following high-dose bolus administration of Compound A in corn oil correlates with the development of hepatic neoplasms in mice exposed to Compound A administered in corn oil. Release of liver enzymes into serum, enhanced labeling indices in hepatocytes, and clear signs of cytotoxicity have been observed in mice exposed by corn oil gavage above 60 mg/kg. In initiation-promotion studies, Compound A showed no initiating or co-carcinogenic activity, although when dosed in corn oil gavage, it appeared to promote liver tumor development in some assays.

Framework for Human Health Risk Assessment Colloquia Series

Colloquium #1

CASE STUDY "B"

1.0 INTRODUCTION

Compound B is produced and widely used as an intermediate in chemical synthesis and in other specialty uses. It dissolves easily in water and is a gas under ambient conditions. In occupational settings, Compound B is sometimes used as an aqueous solution to which workers may be exposed. Inhalation is considered to be the most important route of exposure for Compound B. The compound is a reactive electrophilic species that adducts cellular nucleophiles including DNA and proteins. It also is a metabolite formed in the body from chemicals derived from endogenous and exogenous sources.

Compound B causes a range of effects in humans, including irritation of the eye, skin, and mucous membranes and neurotoxicity. Animal studies have demonstrated cancers of several sites, reproductive and developmental toxicities, lymphocytic necrosis, and kidney toxicity.

This case study focuses on cancer and reproductive/developmental effects associated with Compound B, primarily for inhalation exposures.

2.0 TOXICOKINETICS

Compound B is well absorbed from the respiratory tract, but its reactivity may limit distribution from some exposure sites. Once in the blood, the compound can distribute throughout the body with little apparent selectivity for any tissue (i.e., partitions into all tissues about equally).

The reactive parent is removed by reaction with cellular nucleophiles, metabolism, or exhalation. Alkylation products (adducts) of the reaction of Compound B with blood proteins, including hemoglobin, can be readily followed in humans and animals, providing an internal measure of exposure from both endogenous production and exogenous sources. A number of DNA adducts have been identified and can be measured in DNA from readily collected white blood cells or from internal tissues. Formation of DNA adducts in rat tissues is linear over the range 1 to 30 ppm for 6 hours. The metabolic pathways reduce the chemical's reactivity by hydrolysis or by conjugation with glutathione. Inhalation exposures lead to dose dependent depletion of glutathione at sufficiently high concentrations (e.g. 20% depletion at 100 ppm for 4 hr and 60 to 70% depletion at 600 ppm for 4 hours). Urinary metabolites are derived from the oxidative and glutathione conjugation processes; the spectra of metabolites observed varies quantitatively across species.

3.0 EFFECTS IN HUMANS

Effects associated with humans exposed to Compound B are generally qualitatively consistent with those observed in animals, although available studies are generally limited and inconclusive for reasons including small cohort size and uncertainties about exposure levels.

3.1 Cancer Effects

Some epidemiological studies of workers exposed to Compound B have indicated elevated leukemia, stomach and pancreatic cancers, and Hodgkin's disease, although exposure levels are uncertain. Other studies revealed no excess in these cancers.

3.2 Reproductive/Developmental Effects

Studies of occupationally exposed women have reported mixed results for increased incidences of spontaneous abortion. Estimated, not measured, exposure levels associated with adverse outcomes in one study ranged from 0.1 to 0.5 ppm, with peaks up to 250 ppm.

3.3 Other Noncancer Effects

Exposure to high concentrations of Compound B gas is irritating to the eyes, while exposure to aqueous solutions can produce injury to the eyes and skin. Reports of respiratory effects (e.g. bronchitis) in workers with different exposures are mixed. Central nervous systems effects are frequently reported, including heachache and nausea. Other studies have reported peripheral neuropathy, impaired hand-eye coordination, and memory loss.

4.0 EFFECTS IN ANIMALS

4.1 Cancer Effects

Chronic studies have reported increases in cancer in rats and mice exposed by the inhalation, oral, and injection routes. Oral (7.5 and 30 mg/kg/day) and injection exposure produced dose-dependent increased tumor incidences at local exposure sites but not internal tissues, perhaps indicating that the compound reacted with cellular constituents at the exposure sites and that little systemic distribution occurred. Inhalation exposures produced dose-dependent (33, 100 ppm) increases in mononuclear cell leukemia in females, brain tumors in both sexes, and peritoneal mesotheliomas in male rats that did not survive to study termination. An inhalation study in mice found increases in benign and/or malignant alveolar/bronchiolar and harderian gland tumors in males exposed to 50 and 100 ppm. Females had increases at those two sites and three others, lymphomas, uterine, and mammary tumors.

4.2 Reproductive/Developmental Effects

The reproductive and developmental toxicities of Compound B have been the subject of a number of studies in mice, rats, and rabbits.

Inhalation studies in which both male and females rats were exposed to three concentrations (0,

10, 33, 100 ppm), starting 12 weeks prior to fertilization and continuing through 21 days following parturition, demonstrated effects in the groups with the highest exposure. The gestation period was longer for more females in this group. There were decreases in the number of implantation sites, pups born, and the ratio of pups born to implantations. No effects were observed on parental body weights or organs. A study of Sprague-Dailey rats exposed to 0 or 150 ppm found decreases in maternal body weight, increases in resorptions per litter, and increase in resorptions per implantation site in a group exposed for 3 weeks prior to mating and on days 1 to 16 of gestation. Decreased fetal weights and lengths and reduced ossification of the sternebrae and skull were observed in this group. Another inhalation study with Sprague-Dailey rats on days 6 to 15 of gestation looked at the effects of single or repeated short (1 x 0.5 hour, 3 x 0.5 hour) exposures to high concentrations. Decreased fetal weight was observed following repeated exposure to 800 and 1,200 ppm. Because these studies included exposures during gamete development, fertilization, and fetal development, they do not identify periods of sensitivity to Compound B-induced effects.

Studies of effects on sperm: A variety of studies have shown that sperm abnormalities and genetic changes, including dominant lethal mutations and heritable translocations, occurred in post-meiotic stages of sperm development. No effects were apparent in stem cells from which sperm develop. Studies in mice exposed to 200 or 400 ppm for 5 days by inhalation found increased frequencies of sperm abnormalities.

Dominant lethal mutation is determined by exposing males, mating them with unexposed females, and determining if fetal survival is affected. Inhalation studies in mice exposed to 0, 300, 400, or 500 ppm for 4 days found dose-dependent increase in dominant lethality, though 300 ppm was considered a slight effect. Another inhalation study in mice using 0, 300, 600, and 1,200 ppm for varying times (maintaining a total 1800 ppm-hour exposure) showed a dose rate effect; i.e., increased incidence with short exposure to a high dose rather than equal incidence for all groups. An upward curved dose-dependent increase in dominant lethal effects and heritable translocations has been observed in mice exposed for an extended period (8.5 weeks) to 165, 204, 250, 300 ppm. A dominant lethal effect was observed in offspring of male rats exposed to 1,000 ppm for 4 hours. The mechanism for these effects is not resolved, as stage-specific alkylation of specific proteins have been demonstrated, as well as alkylation of DNA. Although the literature tends to describe these as mutually exclusive options, this may not be the case.

Studies of effects on ova: Limited studies with Compound B indicate that exposure of females can result in altered pregnancy outcomes, likely due to genetic changes in oocytes. Studies with a related chemical have shown that transfer of oocytes to an unexposed mother does not alter the increased incidence of fetal deaths or externally abnormal fetuses.

Studies of fertilized egg (zygote) effects: Studies using inhalation (1,200 ppm) and ip injection (125 mg/kg) have demonstrated increases in fetal death and abnormalities among surviving fetuses when pregnant females are exposed shortly after conception. These effects are highly specific for particular developmental stages (e.g., inhalation exposures at 1 and 6 hours produced effects, while marginal changes were seen with exposures at 9 and 25 hours post-fertilization). The malformations observed were varied. Hydrop and eye defects were the major anomalies observed on day 17 of gestation among offspring of mothers exposed 1 and 6 hour post-fertilization. Other defects were small fetal size, cleft palate, and cardiac, abdominal wall, or extremity and/or tail

defects. Deaths occurred from near the time of implantation until day 17 of gestation when exposure occurred near fertilization. Injection (ip 125 mg/kg) 3 hour post-mating caused fetal deaths and cleft sternum. Skeletal defects due to zygotic exposure differ in kind from those following exposure during organogenesis. The mechanism for these effects is not clear because cytogenetic studies failed to show either structural or numerical chromosome aberrations.

Studies of organogenesis effects: Several studies have demonstrated that exposure during organogenesis can cause fetotoxicity and malformations. In a study in which F344 rats were exposed to 0, 10, 33, and 100 ppm on days 6 to 15 of gestation, no gross external abnormalities were observed. However, the high dose group had reduced fetal body weights and variations in ossification of vertebrae. Two groups of Sprague-Dailey rats exposed to 0 or 150 ppm on days 7 to16 of gestation and days 1 to 16 of gestation had reductions in fetal weights and lengths and decreased ossifications of the sternebrae and skull. Maternal toxicity was observed in the highest dose groups in CD1 mice dosed intravenously with 0, 75, and 150 mg/kg for three days beginning on day 4, 6, 8, or 10 of gestation. Mean fetal weights were reduced 20% in the high- dose groups, as were increased incidences of skeletal malformations. Studies in rabbits dosed intravenously during gestation found dose-related trends for decreased numbers of live fetuses per litter and increased resorptions when dosed days 6 to 14 of gestation.

5.0 ADDITIONAL DATA RELEVANT TO MODE OF ACTION

5.1 Genotoxicity

Much data exist in the literature on the genotoxicity of Compound B using *in vitro* and *in vivo* systems, representing a wide range of prokaryotic and eukaryotic species. Resulting genetic damage includes formation of mutations, specific DNA adducts, increased micronuclei formation in mice and humans, and increased sister chromatid exchanges in peripheral lymphocytes of rats, rabbits, monkeys, and humans. Compound B is clearly a potent mutagenic, alkylating agent whether formed *in vivo* or from exogenous exposure.

5.2 Other Alkylation Targets

Compound B readily alkylates proteins, lipids, RNA, glutathione, and other small molecules present intracellularly or in bodily fluids (e.g. albumin and hemoglobin in blood). The relative importance of adduction of these other cellular molecules as compared to DNA remains an unresolved question. For instance, Compound B alkylates specific proteins in sperm responsible for maintaining DNA integrity. This protein alkylation occurs at those germ-cell stages that are sensitive to Compound B-induced toxicity.

Framework for Human Health Risk Assessment Colloquia Series

Colloquium #1

CASE STUDY "C"

1.0 INTRODUCTION

Compound C is a volatile halogenated hydrocarbon liquid classified by EPA as a Category 3 gas (relatively stable and low water solubility). It has been widely used as an industrial solvent and anesthetic and is a common ground water contaminant.

Compound C was used as an anesthetic due to its ability to depress central nervous system functions; sporadic reports of liver toxicity in humans were associated with this use. Acute nervous system toxicities are observed in animals. Results of epidemiological studies have been controversial with some studies suggesting increased cancer incidences while others do not; noncancer endpoints have not been well studied. The major findings reported in chronic animal studies include kidney and liver toxicity and carcinogenicity, and neurological effects. These effects will be the focus of this case study.

2.0 TOXICOKINETICS

Compound C is rapidly absorbed by the inhalation and oral pathways. Exhalation of unmetabolized Compound C is a major dose-dependent excretory pathway for both oral and inhalation exposures. Compound C is metabolized by a major oxidation pathway catalyzed by cytochrome P450 2E1 and a minor glutathione conjugation pathway, both primarily in liver. The product of the oxidative pathway is an aldehyde which spontaneously adds water, CAL. CAL is reduced to an alcohol (COH) which is conjugated and eliminated in urine. This conjugate is subject to varying amounts of enterohepatic recirculation in different species. CAL can also be oxidized, forming CCOOH. The haloacid is excreted in urine along with other minor metabolites. The glutathione conjugation pathway involves several steps leading to formation of a cysteine derivative, CCYS. CCYS can then be conjugated and excreted in urine or metabolized to a reactive species.

Qualitatively, the pathways are similar across humans, rats, and mice, but quantitatively there are substantial differences. Mice metabolize Compound C very rapidly (more than predicted by body weight scaling), while humans clear CCOOH relatively slowly. Significant interindividual metabolic variations have been observed in humans given a single dose of Compound C or CAL as indicated by urinary excretion of CCOOH ranging from 5 to 50% of the oral dose. Induction of 2E1 by ethanol is observed, although at low concentrations Compound C metabolism is perfusion limited and the increased metabolic capacity will not increase the amount of Compound

C metabolized. In addition, there are differences in the extent of CCYS formation, and the subsequent split between conjugation and formation of reactive species. A polymorphism of the glutathione transferase is known to exist in humans; about 10% of the population is lacking this particular isoform.

3.0 EFFECTS IN HUMANS

3.1 Liver Toxicity and Carcinogenicity

Liver toxicity (noncancer) has been sporadically reported following anesthesia, occupational use, or accidental/intentional ingestion in medical case reports, but it is unclear if other factors were primarily responsible (e.g. preexisting disease). Several epidemiological studies reported no statistically significant increased liver cancer risks in workers exposed to Compound C. A review panel, however, judged that available data in aggregate indicates a slight increase in biliary/liver tumors.

3.2 Kidney Toxicity and Carcinogenicity

Kidney toxicity (noncancer) has been reported sporadically in humans. Studies at one factory where workers were frequently exposed to high concentrations have found tubular degeneration and increases in kidney carcinomas. Concentrations were not measured, so estimates of possible concentrations have been based upon reports of neurological effects such as dizziness. Several other well-conducted epidemiological studies of workers exposed to lower concentrations have found no increase in deaths due to kidney cancer.

3.3 Neurotoxicity

Neurological effects are associated with exposures to a wide range of concentrations of Compound C in air. Anesthesia required approximately 2,000 ppm. Controlled studies with volunteers exposed for short times (hours) found neurological effects including sleepiness, reductions in motors skills, and altered rates of breathing and heart beat. One study (200 ppm for 7 hours for 5 days) reported mild fatigue and sleepiness. Another study (27 and 81 ppm for 4 hours) reported a slight trend toward slower pulse rate. A third study (200 ppm for 2.5 hours) found no effect on heart beat or breathing rates. A fourth study (110 ppm for 8 hours) found decreased performance on skills tests. Controlled studies with exposure to the metabolites, CAL and COH, report similar effects.

4.0 EFFECTS IN ANIMALS

4.1 Liver toxicity

Liver toxicities observed in acute and subchronic studies in mice and rats included increased liver weight to body weight ratio, hypertrophy, small increases in serum levels of liver enzymes, and limited necrosis. These effects were dose dependent both for severity and incidence over dose ranges of approximately 50 to 2,000 mg/kg/day (by oral gavage) and 25 to 600 ppm (by

inhalation). Chronic studies in multiple rat strains report no significant pathology in liver . Increased hepatocellular adenomas and carcinomas were found in mice chronically exposed to approximately 1,000 and 2,000 mg/kg/day or 300 and 600 ppm. Incidences were much higher following corn oil gavage dosing than inhalation exposure. Tumors were observed only in mice following dosing with CAL and CCOOH. A 37 week study with CCYS exposed mice did not find an increase in liver tumors. Acute liver toxicity is increased by several compounds such as ethanol and phenobarbital, reflecting some combination of increased Compound C metabolism at high doses and alterations in the development of liver toxicity.

4.2 Kidney Toxicity

Kidney toxicity, described as degenerative changes in tubules, has been observed in mice and rats of both sexes following chronic oral or inhalation exposure (mice: 1,000 [LOAEL] and 2,000 mg/kg/day; rats: 50 [NOAEL], 250, 500 and 1,000 mg/kg/day or 100 [NOAEL], 300, 600 ppm). This effect is truly chronic; reexamination of tissues from 90 day exposures found only slight indications of kidney toxicity at doses higher than used in the chronic studies. Incidences were particularly high following high dose corn oil gavage exposure of rats. This kidney toxicity is believed responsible for increases in mortality in these chronic studies. Low (<10%, generally 1 or 2 animals per group of 50) incidences of kidney tumors were reported in five strains of male rats in several studies, with statistical significance achieved only in one. CCYS dosing of mice produced kidney toxicity, but not tumors in a 37 week exposure; no lifetime studies are available in rats or mice.

4.3 Neurotoxicity

Chronic studies reported altered behavioral effects in high dose animals (e.g., mice at 1,000 and 2,000 mg/kg/day; rats at 500 and 1,000 mg/kg/day) exposed orally; no data were reported for inhalation studies. In a 42 day study with rats exposed to 50, 100, and 300 ppm increases in brain waves indicative of sleep occurred with dose as did decreases in heart rate. Similar effects have been reported in animals exposed in acute and subchronic exposures to CAL and COH; no neurological effects are observed following dosing with CCOH.

5.0 OTHER DATA RELEVANT TO MODE OF ACTION

5.1 Genotoxicity

Genotoxicity has been the subject of numerous studies with Compound C, CAL, CCOOH, CCYS, and some other minor metabolites of Compound C. No effects are associated with the parent compound. *In vitro* studies with Compound C including metabolic capability have largely been negative, but some positives or equivocal positives have been reported. Some of these latter studies reflect a mutagenic stabilizer, while others used pure material. Studies of CAL have found it to cause clastogenesis and aneuploidy. Studies with CCOOH have been negative. Finally, CCYS is mutagenic in several *in vitro* assays.

5.2 Liver

Liver effects have been observed following exposures to Compound C, CAL, and CCOOH; liver tumors (hepatocellular adenomas and carcinomas) were observed in mice, but not in rats. These species differences in response reflect quantitative pharmacokinetic differences and differences in pharmacodynamics. The acid (CCOOH) is known to cause a range of effects in liver via the peroxisome proliferator-activated receptor (PPAR). Activation of PPAR by a wide range of compounds leads to pleiotrophic responses in the liver. Early liver effects of Compound C exposure include hypertrophy due in large part to proliferation of the subcellular organelles - peroxisomes, induction of specific cytochromes P450 involved in lipid and xenobiotic metabolism, and a brief period of cell proliferation. These responses occur to a much greater extent in mice than rats. Metabolism of Compound C to the acid is also significantly greater in mice than in rats.

Increases in liver to body weight ratio (due to the cell proliferation and enlargement) follow both inhalation and oral exposures to Compound C. A maximum liver weight is reached with increasing dose or for a single concentration, with increasing time up to about 30 days. Studies with other compounds have demonstrated that peptide factors are produced in response to this growth stimulus and stop the cell proliferation and liver enlargement. A selective environment is created due to the continued presence of the original mitogenic stimulus and the antimitogenic signal. Under these conditions, a subsequent genetic change allowing a cell to escape the antiproliferative signal will permit it to proliferate in response to mitogenic stimulus. A number of commonly used human pharmaceuticals activate PPAR, but the pleiotrophic responses observed in rodents with CCOOH do not appear to occur in humans exposed to these PPAR inducers. Structural characteristics of PPAR differ between mice, rats, and humans and PPAR expression is lower in humans.

5.2 Kidney

An extensive database with related compounds and metabolites, including CCYS, has demonstrated that metabolites of CCYS can lead to kidney toxicity, such as tubular degeneration.

Several aspects of kidney disease in exposed factory workers have been studied. Among those workers with kidney cancer, all had varying degrees of tubular damage. Comparable kidney cancer patients without high exposures to Compound C showed tubular damage in about a half of the cases. Alterations in a kidney-specific tumor suppressor gene were observed in 100% of the Compound C exposed workers while these alterations were observed in 33 to 55% of those with kidney cancer but not exposed to the chemical.

5.3 Neurotoxicity

Studies with the metabolites CAL and COH, as mentioned previously, have demonstrated acute or subchronic effects in humans and animals; animal studies reported no effects following CCOOH dosing. An analysis of studies using Compound C or COH was carried out to evaluate potential internal dose metrics in relationship to observed effects. The 42 day animal study showed a nonlinear (curved) dose response curve when altered brain waves or heart beat were plotted versus Compound C exposure dose or estimated peak blood concentrations of Compound C. Versus peak blood concentrations of COH, the response gave a straight line, one indicator of a direct dose response relationship. Analysis of the controlled human studies found peak COH concentrations similar to or greater than those in the 42 day animal study.

Framework for Human Health Risk Assessment Colloquia Series

Colloquium #1

CASE STUDY "D"

1.0 INTRODUCTION

Compound D is a gas at ambient temperatures and is soluble in water. It is a major industrial chemical intermediate for synthetic purposes and arises from bacterial breakdown of related compounds in the environment.

Compound D causes nonneoplastic, preneoplastic, and neoplastic changes in liver which are the focus of this case study; effects in other target organs occur at higher doses.

2.0 TOXICOKINETICS

Human and animal data indicate that Compound D is rapidly and efficiently absorbed via the inhalation and oral routes, rapidly converted to water-soluble metabolites, and rapidly excreted.

Compound D is metabolized mainly by the liver and, at low concentrations, metabolites are excreted primarily in urine. At high exposure concentrations, unchanged Compound D is also eliminated in exhaled air. Overall, the data indicate that neither Compound D nor its metabolites are likely to accumulate in the body.

The primary route of metabolism of Compound D is by the action of the cytochrome P450 2E1 on Compound D to form an epoxide (DO). DO is a highly reactive, short-lived epoxide that rapidly rearranges to form an aldehyde (DALD), also a reactive compound. Metabolite DO is also a substrate for epoxide hydrolase. These two metabolites are detoxified mainly via glutathione (GSH) conjugation.

3.0 EFFECTS IN HUMANS

3.1 Cancer effects

Several independent retrospective and prospective cohort studies demonstrate a statistically significant elevated risk of liver cancer, specifically angiosarcomas, from exposure to Compound D. Liver angiosarcomas are an extremely rare tumor, with only 20 to 30 cases per year reported in the U.S. Since the introduction of the Compound D manufacturing, a significant percentage of reported angiosarcoma cases have been associated with Compound D exposure.

3.2 Histopathological Liver Changes

Occupational studies have also associated Compound D exposure with impaired liver function and/or biochemical or histological evidence of liver damage. Such damage includes hypertrophy and hyperplasia of hepatocytes, activation and hyperplasia of sinusoidal lining cells, fibrosis of the portal tracts and the septa and intralobular perisinusoidal regions, sinusoidal dilation, and focal areas of hepatocellular degeneration.

4.0 EFFECTS IN ANIMALS

Studies in rats, mice, and hamsters administered Compound D via both the oral and inhalation routes indicate liver toxicity. These studies have all reported increased incidences of liver angiosarcomas. Hepatocellular carcinomas have been reported only in rats exposed orally. As described below, altered hepatic foci are observed at low doses, but it is unclear whether to consider these a noncancer effect or simply a very early effects in the cancer process. Other studies have reported increased liver weight and necrosis at relatively high doses compared to the lowest giving rise to cancer.

4.1 Critical Studies in Rats

Wistar rats were administered diets containing 10% polyCompound D with varying proportions of Compound D monomer. Diets were available to experimental animals for 4 hours per day and food consumption and Compound D concentrations were measured at several times during the feeding period in order to account for loss of Compound D from the diet due to volatilization. This information was used to calculate the ingested dose. Evaporative loss averaged 20% over 4 hours. The ingested dose was adjusted downward by the amount of Compound D measured in the feces to arrive at the bioavailable doses of 0, 1.7, 5.0, or 14.1 mg Compound D/kg/day which were fed to Wistar rats (n = 80, 60, 60, and 80, respectively) for a lifetime. An additional group of 80/sex were administered 300 mg/kg bw/day, by gavage in oil for 5 days/week for 83 weeks. Rats were weighed at 4 week intervals throughout the study. Hematological values were obtained at 13, 26, 52, 78, and 94 weeks, and blood chemistry was performed at 13, 26, 52, and 106 weeks (n=10). Urinalysis was performed on 10 animals per group at 13, 25, 52, 78, and 94 weeks. All surviving animals were necropsied at week 135 (males) or week 144 (females). Interim sacrifices of 10 animals at 26 and 52 weeks included animals from the control and high dose group.

There was no difference in body weights in the Compound D treated animals, although all groups (including the control) weighed significantly less than the controls fed ad lib (treated animals had access to food for only 4 hours/day). Significant clinical signs of toxicity in the 5.0 and 14.1 mg/kg/day groups included lethargy, humpbacked posture, and emaciation. Significantly increased mortality was seen consistently in males at 14.1 mg/kg/day and in

females at 5.0 and 14.1 mg/kg/day. No treatment-related effects on hematology, blood chemistry, or urinalysis parameters were observed. Relative liver weight was significantly increased at 14.1 mg/kg/day, but was not reported for the other dose groups.

A variety of liver lesions were observed histologically to be dose-related and statistically significant in male and female rats. These included clear cell foci, basophilic foci, eosinophilic foci, neoplastic nodules, hepatocellular carcinoma, angiosarcoma, necrosis, cysts, and liver cell polymorphism. Several of these endpoints were significantly increased in the group exposed to 1.7 mg/kg/day. Of the above lesions, all except the angiosarcoma derive from hepatocytes; angiosarcoma is derived from sinusoidal cells. The neoplastic nodules, cysts, and altered hepatocellular foci are proliferative lesions indicative of changes in the cells from which hepatocellular carcinomas are derived. However, an ambiguity in the designation of neoplastic nodules should be noted. This study designated the lesions as neoplastic nodules according to the criteria of Squire and Levitt (1975). More recent diagnostic nomenclature adopted by the National Toxicology Program (NTP) uses the terms "hepatocellular adenoma" and "hepatocellular hyperplasia" for the lesions previously diagnosed as "neoplastic nodules". The NTP classification reserves the term hyperplasia for "proliferative lesions that are perceived to be a secondary, nonneoplastic response to degenerative changes in the liver." By contrast, the report states, "foci of cellular alteration, hepatocellular adenoma, and hepatocellular carcinoma are believed to represent a spectrum of changes that comprise the natural history of neoplasia."

Thus, the "neoplastic nodules" observed in this study include both neoplastic and nonneoplastic lesions, and the altered hepatocellular foci are preneoplastic lesions. Consistent with this designation, the foci occur at lower doses and higher incidences than the hepatocellular carcinomas. These lesions occur at doses one to two orders of magnitude lower than other liver lesions. The incidence of necrosis was increased in a dose-related manner that was statistically significant in males at 14.1 mg/kg/day and in females at 5.0 mg/kg/day. Proliferation of sinusoidal cells showed a dose-related increase in males, but did not achieve statistical significance.

This study defines a NOAEL of 1.7 mg/kg/day and a LOAEL of 5.0 mg/kg/day for liver effects that are not thought to be preneoplastic. Increased tumor incidence was noted in all treated groups. Almost exclusively angiosarcomas were observed in males and females administered 300 mg/kg/day by gavage, while a mixture of angiosarcomas and hepatocellular carcinomas was observed at the mid- and high dietary doses. Only hepatocellular carcinomas were reported at the low dose.

The lifetime dietary study was performed in order to study a range of oral doses below that delivered in the previous study, since tumors were observed at all doses in the previous study. The oral doses were delivered in the same way except that the diets contained a final concentration of 1% polyCompound D, rather than 10%. Wistar rats (100/sex/dose) were administered doses of 0, 0.014, 0.13 or 1.3 mg Compound D/kg/day for 149 weeks. Mortality differences were not remarkable for males, but were slightly increased for females receiving 1.3 mg/kg/day. Relative organ weights were not evaluated. Angiosarcomas were observed in one high-dose male and two high-dose females. Other significant increases in tumors were limited to neoplastic nodules in females and hepatocellular carcinomas in males. An increased incidence of basophilic foci was observed in both sexes at 1.3 mg/kg/day and only in females in the two lower dosage groups. Rats exposed to 1.3 mg/kg/day also had a significantly increased incidence of liver cell polymorphism, hepatic cysts, neoplastic nodules, and hepatocellular carcinoma. No increases in nonneoplastic endpoints were observed.

5.0 ADDITIONAL DATA RELEVANT TO MODE OF ACTION

5.1 Genotoxicity

In vitro genotoxicity assays indicate that Compound D is mutagenic, causing point mutations in the presence of exogenous metabolic activation. Similar assays show that the major Compound D metabolite, Compound DO, is positive without metabolism in genotoxicity tests. *In vivo* genotoxicity tests with Compound D also provide evidence of genotoxicity. DNA adducts formed from Compound D metabolites have been identified following both *in vivo* and *in vitro* exposures. These include a major, but short lived metabolite and several minor, but more persistent adducts.

5.2 Role of Metabolites

Compound D must be metabolized to cause toxicity or carcinogenicity. The reactive, short-lived metabolites, Compound DALD and Compound DO, are responsible for the toxic and carcinogenic effects of Compound D. Both Compound DALD and Compound DO can react with tissue nucleophiles, but Compound DALD appears to be the most important source of tissue protein adducts. Compound DO is the reactive metabolite responsible for DNA adducts. In part this difference may result from the ability of the more lipophilic metabolite Compound DO to reach the nucleus, as opposed to Compound DALD which, although it is produced in greater quantities, is too water soluble to cross the nuclear membrane.

5.3 Liver tumorogenesis

Mutations in the p53 tumor suppressor gene are the most common gene alteration identified in human cancers and have been associated with human hepatocellular carcinomas and angiosacrcomas, including those due to exposure to Compound D. *Ras* oncogene mutations have also been found in human liver cancers; Compound D-induced human angiosarcoma is also associated with mutations of *ras* oncogenes. Rodent liver tumor response is more variable in nature. While liver angiosarcoma is a rare tumor in all species, hepatocellular carcinoma has a high spontaneous incidence in some rodent strains. Knockout of the p53 tumor suppressor gene in mice results in the spontaneous development of angiosarcomas, along with malignant lymphomas, but not hepatocellular carcinoma. In contrast, accelerated development of hepatocellular carcinomas in rodents is associated with overexpression of the *myc* and *ras* oncogenes, but not with mutational loss of p53 function. Rat angiosacrcomas due to Compound D exposure show mutations of p53.

Framework for Human Health Risk Assessment Colloquia Series

Colloquium #1

CASE STUDY "E"

1.0 INTRODUCTION

Compound E is a common contaminant found in drinking water. It is an element that exists in a variety of oxidation states, complexes (e.g., oxides), and organic derivatives (e.g., methylated forms). These various forms occur naturally, synthetically, or as byproducts of industrial processes.

A range of external (skin) and internal toxicities have been observed. Oral exposures have resulted in nonneoplastic and neoplastic skin diseases, cardiovascular effects, irritation of the gastrointestinal tract, anemia, and cancers of the lung, bladder, kidney, and perhaps other internal organs. Inhalation exposures have been associated with nonneoplastic and neoplastic changes in the respiratory tract (e.g., lung).

This case study describes chronic toxicities associated with Compound E exposures.

2.0 TOXICOKINETICS

Following exposure, Compound E is well absorbed by the oral and inhalation routes; dermal data is lacking. The two major inorganic oxidation states of Compound E are interconverted in the body. The other major metabolic fate of Compound E is methylation in the liver; methylated forms are the major urinary metabolites. The reduced form interacts with sulfhydryls, particularly neighboring sulfhydryls that result in a 5-membered ring as the product. This is a major contributor to toxicity, although the oxidized form can substitute for phosphorus in a wide variety of endogenous compounds (e.g., ATP) so it may contribute to toxicity.

Metabolic pathways in humans and rodents are qualitatively similar with no striking quantitative differences beyond those associated with typical interspecies differences of scale. Methylation is essentially linearly related to metabolism with increasing dose in humans, though under controlled experimental conditions saturation of methylation can be demonstrated at sufficiently high acute doses. Similar findings occur in mice.

3.0 EFFECTS IN HUMANS

3.1 Noncancer

Ingestion of Compound E by humans is usually not associated with serious injury to the respiratory system, although pulmonary edema and hemorrhagic bronchitis may occur in moderate to severe cases. Insufficient data exist on the exposure levels in these studies to identify a no-effect level for respiratory tract irritation with confidence, but it appears such effects are minor or absent at exposure levels of about 0.1 to 1 mg/m^3.

A number of studies in humans indicate that Compound E ingestion may lead to serious effects on the cardiovascular system. Long-term low-level exposures may also lead to damage to the vascular system. The disease is characterized by a progressive loss of circulation in the hands and feet, leading ultimately to necrosis and gangrene. Studies indicate that ingestion of 0.6 to 0.8 ppm Compound E in drinking water (corresponding to doses of 0.02 to 0.06 mg/kg/day, depending on age) leads to circulation changes. Workers exposed to Compound E dusts may also have an increased incidence of Raynaud's disease and an increased constriction of blood vessels in response to cold at exposure levels above about 0.05 to 0.5 mg/m^3.

Anemia and leukopenia are common effects of Compound E poisoning in humans, and have been reported following acute and chronic oral exposures. Hematological effects are usually not observed in humans exposed to levels of 0.07 mg/kg/day or less, although intermediate-duration exposure to 0.05 mg/kg/day resulted in mild anemia in one study. Although anemia is often noted in humans exposed to Compound E by the oral route, red blood cell counts are usually normal in workers exposed by inhalation. The reason for this apparent route specificity is not clear, but might simply be related to dose.

A number of studies in humans exposed to inorganic Compound E by the oral route have noted signs or symptoms of hepatic injury. Clinical examination often reveals that the liver is swollen and tender, and analysis of blood sometimes shows elevated levels of hepatic enzymes. These effects are most often observed after chronic exposure to doses of 0.019 to 0.1 mg/kg/day. Histological examination of the livers of persons chronically exposed to similar doses has revealed a consistent finding of portal tract fibrosis leading in some cases to portal hypertension and bleeding from esophageal varices. Hepatic toxicity has not been investigated in humans following inhalation exposure.

One of the most common and characteristic effects of Compound E ingestion is a pattern of skin changes that include generalized hyperkeratosis and formation of hyperkeratotic warts or corns on the palms and soles, along with areas of hyperpigmentation interspersed with small areas of hypopigmentation on the face, neck, and back. These effects have been noted in a large majority of human studies involving intermediate- or chronic-duration oral exposure. Numerous studies in humans have reported dermal effects at chronic dose levels ranging from about 0.01 to 0.1 mg/kg/day. Dermal effects are usually not mentioned in studies of persons exposed primarily by inhalation.

3.2 Cancer

Most epidemiological studies of Compound E carcinogenicity focus on populations drinking Compound E-containing waters or workers exposed occupationally by inhalation of smelter dusts. Other groups studied have included residents living near industrial releases, occupational cohorts, and humans treated medically with Compound E. Chronic oral exposures increased the risk of developing skin cancer and cancers of some internal organs; inhalation exposures increased risk for lung cancer.

The most widely studied location had well water containing Compound E concentrations ranging from 0.01 to 1.82 mg/L. The population in this area largely shared similar socioeconomic status and living conditions, including medical care, so that variations in Compound E levels were the only apparent major environmental difference. The study population was classified into four groups, according to concentrations in the wells: <0.1 ppm (13 towns), 0.1 to 0.29 ppm (8 towns), 0.3 to 0.59 ppm (15 towns), and greater than 0.6 ppm (6 towns). In this area, 10.6 people per 1000 were found to have skin cancer. The male to female ratio was 2.9 to 1 and the prevalence of skin cancer increased with increasing Compound E concentration in drinking water. Using age-adjusted mortality rates of this same population, significant dose-responses have been reported between Compound E levels in well water and mortality from several cancers. Skin, bladder, kidney, and lung cancers were reported most consistently while cancers of the nasal cavity, colon, liver, and prostate have been less frequently identified.

Some uncertainty exists concerning the quantitative comparability of the population of this area with others throughout the world. For instance, Compound E-induced skin cancer prevalence in the residents was increased by other risk factors including liver dysfunction among carriers of hepatitis B surface antigen and dietary factors. The liver cancers observed have been suggested to indicate interactions between hepatitis B, aflatoxin, and Compound E. Other potential risk factors that have been raised, but for which data are not always available include the oxidation state of the inorganic Compound E, the presence of other disease states whether Compound E-induced or not, smoking, and the length of exposure. Thus, studies from other populations exposed orally are of significant interest.

Epidemiological studies of drinking water exposure have been reported in other locations around the world. Findings of skin cancer or internal cancers have been mixed reflecting differences in many factors, including population size studied, drinking water concentrations, length of exposure, and length of time since exposure (latency period).

Two towns in a second location were compared with regard to Compound E levels in drinking water. The well for the exposed population was found to contain 0.41 mg/L, while the well in the control town had an Compound E concentration of 0.007 mg/L. Increased incidences in skin pigmentation were found. Of the exposed individuals found to have pigment alterations, 1.4% had ulcerative zones classified as skin cancer, but a statistically significant excess incidence of skin cancer has not been reported. Recent studies in these populations have measured chromosomal alterations in blood cells and found higher incidences among those with Compound E exposure compared to a control population.

Another cohort, comprised of individuals exposed to well water containing Compound E

concentrations greater than 1 mg/L for about 5 years, was reported to have an increased observed standard mortality ratio for both lung and urinary tract cancer, relative to expected mortality. This study also suggests synergism between oral Compound E intake and smoking for the development of lung cancer.

A study in another location evaluated 20,000 residents who were exposed long-term to drinking water that contained Compound E concentrations estimated at 0.17 to 0.33 mg/L as compared to a similar number of people with very little exposure. No significant differences in peripheral vascular disorders, peripheral neuropathy, or cancer frequency were observed. Studies of populations in a another location exposed to drinking water containing Compound E have been negative for skin cancer and internal cancers. Among residents in one region, no correlation was found between Compound E levels in drinking water and incidence of skin cancer. In this study, only 5% of water samples contained 100 mg/L or more as compared to 48% of the samples in the first studies described above which were positive. Another study evaluated the association between Compound E intake, which ranged from 0.0005 to 0.16 mg/L (mean 0.005 mg/L), and bladder cancer. No relationship was found between bladder cancer and either cumulative Compound E exposure or intake concentration. An ecological study of skin cancer cases did not find an increased incidence in the two counties presumed Compound E-exposed as compared to the control counties. No water concentrations are reported in this study. Several other studies from this country are also available with similar findings.

Although medicinal exposures to Compound E are not identical to drinking water exposures, studies of this population provide other data on cancer following oral intake. Cancers of the skin and internal organs have been reported. A significant excess incidence of fatal bladder cancer and a weak dose-response trend for respiratory cancer have been reported among treated with Compound E for periods ranging from 2 weeks to 12 years. It was also noted in these studies that among a group of patients examined for dermatological signs of Compound E exposure, all cancer deaths occurred among those showing evidence of skin disease.

Inhalation of Compound E dusts represents the other major route of exposure. Studies of several worker populations who were exposed to Compound E via inhalation, reported associations between occupational Compound E exposure and increased lung cancer mortality rates. One study established a dose response for increased respiratory cancer using categorization of low ($<100\ \mu g/m^3$), medium (100 to 499 $\mu g/m^3$), high (500 to 4,999 $\mu g/m^3$) and very high (5,000 $\mu g/m^3$). Two studies used the multistage model of carcinogenesis to analyze inhalation data. In both cases, the effects of Compound E were found to likely be at late stages of the cancer process.

4.0 EFFECTS IN ANIMALS

Carcinogenicity of Compound E has been extensively studied in laboratory animals. Cancers did not result except in some studies with methylated forms following dosing with a genotoxic chemical as initiator (i.e. initiation-promotion protocol). Noncancer effects have been less extensively studied in laboratory animals. Histopathological observations of gastrointestinal irritation, blood alterations, dermal effects, and other noncancer effects observed in humans have generally not been seen with chronic exposure of rodents. Little data are available for some

potential effects such as reproductive or developmental toxicities.

5.0 ADDITIONAL DATA RELEVANT TO MODE OF ACTION

5.1 Genotoxicity

Results from *in vitro* mutagenicity tests of Compound E with both bacterial or mammalian cells indicate that Compound E alone is either an inactive or extremely weak mutagen. Concentrations of Compound E that were weakly mutagenic were also cytotoxic.

Compound E has been reported to be a comutagen, enhancing the mutagenic response to ultraviolet (UV) light in E.coli, UV and methyl methanesulfonate (MMS) in Chinese hamster ovary (CHO) cells, and with N methyl-N-nitrosourea (MNU) in V79 cells. Clastogenic effects, such as sister chromatid exchanges (SCEs) and chromosomal aberrations, have also been observed following administration of Compound E compounds to mammalian cells in vitro. These aberrations were observed over the same concentration range for which cell transformation was observed, with the reduced form being more active than the oxidized form. SCEs were also observed for both chemicals. These types of clastogenic effects have also been observed in human cells following treatment with Compound E as have DNA-protein crosslinks.

No oncogene or tumor suppressor gene changes have been clearly associated with Compound E-induction of human tumors, though there is one report of an unusual spectra of mutational changes in the p53 gene in bladder tumors from Compound E exposed individuals. Alterations in this tumor suppressor gene are common late in human tumor processes, but are rare in rodent tumors. p53 plays a role in the check function for cell replication at the G1 → S checkpoint preventing replication of cells with DNA damage.

A study examined the frequency of lymphocyte chromosomal aberrations and of micronuclei in exfoliated oral mucosal or urothelial cells from residents of towns with low or high Compound E exposure. A significant increase in the frequency of lymphocyte chromosomal aberrations, consisting of chromatid or isochromatid deletions, was reported in the population with high Compound E compared to that with low exposure. Also, a significant increase in the frequency of micronuclei in oral mucosal epithelial cells or urothelial cells was observed.

5.2 Observations Focused On Proteins

Compound E is highly reactive with peptide and protein sulfhydryl groups. However, it is now known that Compound E can also be selective in this process, reacting with only a small number of closely spaced dithiol groups. One target is lipoic acid, a cofactor for pyruvate dehydrogenase involved in mitochondrial production of acetylCoA. Others include proteins important to DNA repair.

Compound E compounds, therefore, could cause or potentiate chromosomal damage by interfering with DNA repair enzymes. Different mechanisms may be responsible for the induction of chromosomal aberrations and SCEs. Restriction endonucleases that induce only DNA double-strand breaks have been shown to induce chromatid exchanges and deletions, but not SCEs. *In vitro* studies of DNA ligases (which contain closely spaced dithiols) involved in excision repair have shown that Compound E is a selective inhibitor of one of the two ligases present in Chinese hamster V79 cells. When these cells were treated with Compound E, no radiolabeled CTP was incorporated; following MNU treatment, which causes single strand breaks, dCTP was incorporated and then removed indicating DNA repair had occurred. When cells were treated with MNU followed by Compound E, there was no decrease in radiolabeled dCTP indicating repair had been inhibited. Further studies determined that DNA ligase II was involved. Decreases in the activity of alkyltransferase proteins, involved in removing alkylated bases from DNA, have been found with Compound E but were not dose dependent. Compound E is also known to induce expression of so-called heat shock proteins. These proteins play a variety of roles in cellular responses to stress; Compound E-protein complexes may appear "denatured" and induce this stress response.

Gene amplification can be an important process in carcinogenesis that can arise from chromosomal instability and recombination initiated by unrepaired single-strand breaks. Compound E treatment of mouse 3T6 cells results in a dose-dependent increase in colonies made methotrexate-resistant by amplification of the dihydrofolate reductase gene. The difficulty in detecting Compound E carcinogenicity in animals may be related to its ability to cause gene amplification, but not gene mutations. Amplification of an altered or activated oncogene may occur in a late stage of carcinogenicity and induction of this process could increase the incidence of tumors.

5.3 Oxidative Damage

Metabolic formation of free radicals and the production of oxidative stress may contribute to the toxicity of Compound E. In a series of experiments, the effects of Compound E on cultured human skin fibroblasts, CHO cells, and E-resistent cell lines were studied. The CHO cells were 10-fold less sensitive to acute toxicity than the human skin fibroblasts. Treatment with Vitamin E, an antioxidant, was partially protective in fibroblasts, but had no effects on CHO cell survival. Sensitivity to oxidative damage may be a function of cellular antioxidant capabilities which were greater in CHO than fibroblast cells. Management of oxidative stress may explain differential cell toxicity; some resistant cells have higher levels of heme oxygenase which may act by reducing cellular heme pools and thereby reduce oxygen radical formation.

Increases in single DNA strand breaks were observed in lungs of male ICR mice administered 1,500 mg/kg of a metabolite of Compound E. No increases were observed in several other tissues including liver and kidney. Because of the elution pattern, strand breakage was assumed to be caused by a free radical of this metabolite. Furthermore, clumping of heterochromatin in the nuclei of endothelial cells of the alveolar wall capillaries in these mice was attributed to radicals.

5.4 DNA Methylation

Methylation of DNA plays a major role in regulation of gene expression, both in normal tissues

and in preneoplastic and neoplastic tissues. Because Compound E is detoxified via methylation, its metabolism might alter DNA methylation which are dependent upon the same enzymes (methyltransferase) and methyl donor molecules (S-adenosylmethionine). Exposure of human lung adenocarcinoma cells to Compound E with two different oxidation states, but not a methylated metabolite of Compound E, produced significant dose-response hypermethylation in the promoter region of the p53 tumor suppressor gene. This was determined by restriction mapping and sequencing. Limited data also suggest that hypermethylation may exist over the entire genome in response to exposure of these cells to Compound E.